High dopaminergic D2 receptor availability as assessed by ^{11}C-raclopride PET is associated with appetitive aggression in Long Evans rats

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CHAPTER 6

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Abstract

Background: Violence and appetitive forms of aggression are serious concerns for modern society**.** Rewarding properties of winning aggressive encounters reinforce aggressive behaviour through instrumental learning, and dopamine (DA) receptors within the nucleus accumbens (NAc) are implicated in these natural rewards of positive behavioural outcomes.

Objective*:* To assess DA D2 receptor availability in the striatum of winning experience-enhanced aggressiveness in Long Evans (LE) rats.

Methods: Male outbred LE rats (n=16, 4 months-old) were screened for aggression levels and their capacity to defeat an intruder rat in the resident-intruder paradigm. Based on the tendency to initiate attacks (attack latency; AL <1 min) and effectiveness to subjugate intruders, rats were assigned to an aggressive (n=10) and a non-aggressive group (n=6). Aggressive rats were further used as residents to successfully defeat intruders with an average of 14±5 aggressive confrontations per rat. At the end of the study, both aggressive and non-aggressive rats underwent 60-min dynamic PET scans with the dopaminergic D2 antagonist ^{11}C-raclopride for quantification of D2 receptor availability in the NAc, nucleus caudates/putamen (CPu) and cerebellum.

Results: During aggression screening, the AL of aggressive rats was 45s, IQR 40-83 s *vs.* 123s, IQR 66-461s in non-aggressive rats, p=0.010). Upon repetitive winning aggressive encounters, the aggressive rats showed a decrease in AL of 1.8s after each victory (p=0.006). ^{11}C-raclopride binding potential (BP_{ND}) was higher in the NAc and in the caudate and putamen (CPu) of aggressive rats, as compared to non-aggressive (1.14, IQR 1.01–1.28 *vs.* 0.83, IQR 0.77–1.03, p=0.007; and 2.26, IQR 2.23–2.40 *vs.* 1.98, IQR 1.66–2.09, p<0.001; respectively). Moreover, the AL of aggressive rats was negatively correlated with the BP_{ND} in the NAc (r_s=-0.720, p=0.019) but not in the CPu.

Conclusions: For the first time we were able to demonstrate through ^{11}C-raclopride PET that aggressive rats exposed to repetitive winning confrontations display higher levels of D2 receptors, when compared to non-aggressive rats. The negative correlation between the AL and BP_{ND} in NAc of aggressive rats suggests that an aggression habit might be developed by the winning reward feeling through stimulation of the dopaminergic system. However, future research is needed to corroborate and further explore our current findings.

Key words: aggression, dopaminergic system, resident-intruder paradigm, PET.

Introduction

It is commonly accepted in biology that aggression is one of the most widespread and functional forms of social behaviour that ultimately contributes to fitness and survival of individuals. Clearly, aggression is the behavioural weapon of choice for essentially all animals and humans to gain and maintain access to desired resources (food, shelter, mates), defend themselves and their offspring from rivals and predators, and establish and secure social status/hierarchical relationships. However, aggressive behaviour can transition from adaptive to maladaptive. A relatively small proportion of individuals may show excessive/inappropriate aggressive behaviours and/or can become extremely violent. This escalated aggression and violence is a major source of death, social stress and ensuing disability, thereby constituting one of the most significant problems for the public health, medical institutions and criminal justice systems worldwide. In order to reduce violent and inappropriate forms of aggressive behaviour, more fundamental knowledge on the determinants of aggression is greatly needed. Much evidence suggests that the interaction between environmental factors and neurochemical substrates is instrumental in escalated and maladaptive forms of aggression (1). Since these interactions are difficult to investigate in humans, experimental laboratory animal models of aggression are necessary.

To date, most laboratory animal studies of aggression are employing the resident-intruder aggression paradigm using highly domesticated rodent species like mice and rats that generally are very placid and docile. In virtually all laboratory inbred/outbred mouse and rat strains, the aggressive behavioural traits have been dramatically compromised due to selection and inbreeding during the course of the domestication process (2). Consequently, in order to promote appreciable levels of aggression in these laboratory animal strains, several procedural manipulations are being employed. One way to increase aggressive tendencies is by providing animals with repeated positive (i.e., winning) aggressive experiences in its home cage. Numerous studies in a wide variety of animal species have convincingly demonstrated that in addition to securing access to resources, the most intriguing consequence of winning aggressive conflicts is the self-reinforcing effect of this type of behaviour. Actually, individuals seek out the opportunity to fight and engaging in aggressive behaviour appears to be a source of pleasure, referred to as “appetitive” aggression (3). The most convincing evidence that successful aggression seems rewarding to animals is that the opportunity to engage in aggressive behaviour can reinforce operant responding for future aggression (see Miczek et al., 2004

for review (4)) and induce conditioned place preference for a location associated with a previously successful aggressive encounter (5).

Not surprisingly, just like other events that function as positive reinforcers such as food, drugs or sex, the mesocorticolimbic dopamine system is closely associated with the rewarding properties of winning fights. Nucleus accumbens (NAc) dopamine is strongly released during anticipation of aggressive episodes (7) and pharmacological antagonism of dopamine D1/D2 receptors in the NAc diminishes the seeking of the opportunity to fight (9; 11). In addition, direct optogenetic activation of ventral tegmental area (VTA) dopamine neurons increases aggression (13), while DA receptor knock-out mice show a reduced aggressive phenotype (15; 17), proving that dopamine function and aggression are causally linked. Furthermore, DA D2/3 receptor binding was elevated in the nucleus accumbens shell and dorsal striatum of dominant rats when compared to subordinate rats and was accompanied by elevated DAT and reduced dopamine content in the nucleus accumbens shell (22). Similarly, socially-housed dominant monkeys that were engaged in aggressive behaviour had increased levels of D2 receptors in the basal ganglia when compared to subordinates as observed with ^{18}F-fluorocleboperide PET imaging (23). This finding was confirmed in dominant female cynomolgus monkeys (24). Together, these studies provide strong evidence for a role of DA receptors in the ventral striatum in mediating winning experience-enhanced aggressiveness.

To date, no study has investigated the link between the dopaminergic system and aggression levels in rodents through PET yet. Therefore, the aim of this study was to evaluate differences in dopaminergic D2 receptor availability between aggressive and non-aggressive Long Evans (LE) rats using ^{11}C-raclopride PET. Aggressive LE rats have been exposed to repetitive winning confrontations leading to escalated and/or appetitive forms of aggressiveness.

Materials and Methods

Experimental animals

Male outbred LE rats (n=16, 16 weeks old, 518 ± 33g; Harlan, Indianapolis, USA) were used as residents in the present study and divided into two groups based on their level of aggressiveness in the resident-intruder test. All animals were kept under a 12:12 hour light:dark cycle, with lights on at 7 a.m. Rats had *ad libitum* access to food and water.

Animal experiments were performed in accordance with the Law on Animal Experiments of the Netherlands. The protocol was approved by the Institutional Animal

Care and Use Committee of the University of Groningen (protocols DEC 6828A and DEC 6828B).

Study design

The study was divided in three parts. In the first part, the male LE rats were screened for aggression for three consecutive days. Each male rat was housed for fourteen days in large cages (80x50x40 cm) together with a tubal-ligated female LE rat in order to stimulate territorial aggression. Before the aggression test, the female was taken out of the cage before a male Wistar rat intruder was placed inside the resident's cage. The attack latency (AL; used as an indicator of an animal's aggressiveness) of the LE rats and the ability to successfully defeat the intruder (i.e. intruder assuming a submissive posture for at least 3 seconds) during a 10-min interaction were recorded. The rats always encountered an unfamiliar opponent. An AL smaller than 1 min (6) during the training period combined with a successful winning confrontation was defined as aggressive behaviour (8). After screening, rats were divided in aggressive and non-aggressive rats. The aggressive rats were used as residents for the second part of the study, a longitudinal repeated social defeat study (RSD, see (10)). The non-aggressive rats were housed with a female until the PET scan, without further interventions. The third part consisted of ^{11}C-raclopride PET imaging of all male LE rats (non-aggressive and aggressive), at least two weeks after the last winning confrontation to minimize any residual effect of acute dopamine release. No age differences were present between groups during the PET scans.

Repeated social defeat (RSD)

The RSD protocol was conducted as previously described (10). The female companion of the LE resident rat was removed from the cage shortly before the defeat test. The resident rat was confronted with an unfamiliar intruder Wistar rat being placed inside the resident's cage for each aggressive confrontation, in order to prevent habituation. In general, the residents quickly explored the intruder and shortly after performing the threatening repertoire (12), proceeded with the overt clinch attack. Both rats were allowed to interact for a period of 10 min or shorter if the resident was able to successfully defeat the intruder, interpreted as the intruder assuming a supine (submissive) position for at least 3 seconds. After submission, the intruder was placed inside a wire mesh cage to avoid physical contact with the resident, still allowing visual, auditory and olfactory

interactions for a total exposure period of 60 min. The RSD experiment always took place between 16:00 and 18:00 p.m.

Tracer synthesis

^{11}C-raclopride was synthetized by alkylation of S-(+)-*O*-desmethyl-raclopride (ABX, Radeberg, Germany) using ^{11}C-methyl iodide as the reagent (14). ^{11}C-methyl iodide was trapped in a solution containing 1mg of S-(+)-*O*-desmethyl-raclopride and 1.4 mg of sodium hydroxide in 300 µl dimethylsulfoxide. The reaction mixture was allowed to react for 4 minutes at 80°C. After the reaction, the product was purified through HPLC using a µBondapak C18 column (7.8mmx 300mm) and acetonitrile/H_3PO4 10mM (30/70) as the eluent (flow 5 ml/min). To remove organic solvents from the product, the HPLC fraction containing the product (retention time of 8 min) was diluted in 100 ml of water and passed through an Oasis HLB 200 mg cartridge. The cartridge was washed twice with 8 ml of water and subsequently eluted with 0.8ml of H_3PO4 1% in ethanol and 8 ml of phosphate buffer (pH 7.2). The product was sterilized with a 0.20 µm Millex LG filter. The radiochemical purity was always >98% and the molar activity at the end of the synthesis was 163 ± 69 GBq/µmol.

Dynamic PET imaging

PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Rats were anesthetized with isoflurane mixed with oxygen (5% for induction, 2% for maintenance) and the tail vein was cannulated for tracer injection. Rats were placed in the camera in prone position with their head in the field of view. A transmission scan was acquired using a ^{57}Co point source for attenuation and scatter correction. ^{11}C-raclopride (21.04 ± 10.55 MBq; 0.18 ± 0.20 nmol, p=0.22) was injected over 1 min using an automatic injection pump at a speed of 1 mL/min, and a 60-min dynamic PET scan was acquired. The body temperature was maintained at 37°C with heating pads, heart rate and blood oxygen saturation were monitored, and eye salve was applied to prevent conjunctival dehydration.

Image reconstruction and analysis

The list-mode data from the 60-min emission scan were reconstructed into 21 frames (6 x 10, 4 x 30, 2 x 60, 1 x 120, 1 x 180, 4 x 300 and 3 x 600 s). Each emission frame was corrected for radioactive decay, scatter, random coincidences and attenuation, and

reconstructed using the two-dimensional ordered-subset expectation maximization (OSEM2D) algorithm (4 iterations and 16 subsets). Final images had a 128 x 128 x 95 matrix with a pixel width of 0.475 mm and slice thickness of 0.796 mm. PET images were automatically co-registered to a functional ^{11}C-raclopride brain template (16), which was spatially aligned with a stereotaxic T2-weighted MRI in Paxinos Space using PMOD 3.6 (PMOD technologies Ltd., Switzerland). Time-activity curves (TACs) were generated for the caudate and putamen (CPu), NAc and cerebellum by applying the corresponding predefined volume of interest (VOIs) (16) to the dynamic data.

Following the well validated approach for ^{11}C-raclopride, the simplified reference tissue (SRTM) model was applied to quantify tracer uptake (18; 19). The instantaneous changes in tracer concentration in each compartment can be described as:

$$\frac{dC_T(t)}{dt} = K_1^T C_P(t) - k_{2a}^T C_T(t)$$
$$\frac{dC_R(t)}{dt} = K_1^R C_P(t) - k_{2a}^R C_R(t)$$

where $C_P(t)$ is the tracer concentration in plasma, $C_T(t)$ and $C_R(t)$ are the concentration in target and reference compartments, K_1^T and K_2^R are the rate constants describing the tracer influx from plasma to the respective compartments, k_2^R is the reference washout rate from the reference to the plasma, k_{2a}^T is the apparent target washout rate constant and t is time (20). The extracted TACs were fitted to the SRTM using the cerebellum as reference region and the non-displaceable binding potential (BP_{ND}) was calculated for the CPu and NAc.

Statistical analysis

Results are reported as median and the 0.25-0.75 interquartile range (IQR). Statistical analysis was performed using IBM SPSS Statistics 23 software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY). Differences between-groups were analysed by the Mann Whitney U test and considered to be significant when $p<0.05$, without correction for multiple comparisons. The correlations between the BP_{ND} of the investigated brain areas and the AL were assessed through the Spearman correlation (r_s) test.

In order to evaluate if changes in the AL (escalation of aggression) were related to the number of winning confrontations, the generalized estimating equations (GEE) model was applied (21) because of repeated measurements and missing data. The AR(1) working correlation matrix was selected according to the quasi-likelihood under the independence model information criterion value. Wald's statistics and associated *p*-values were considered statistically significant if $p<0.05$.

Results

Attack latency

Based on the averaged AL of the first three screening days, it was possible to identify 6 non-aggressive and 10 aggressive rats (AL of non-aggressive rats: 123 s, IQR 66 – 461 s vs. aggressive rats: 45 s, IQR 40 – 83 s, $p=0.01$). The average AL of all winning confrontations of aggressive rats was 20 s, IQR 4 – 75 s. Aggressive rats were exposed to an average of 14±5 winning encounters.

Upon repeated aggression testing and acquiring victorious experiences, the time to initiate aggressive attacks gradually decreased in the aggressive animals. In aggressive rats, a significant correlation between the number of winning confrontations and the AL was observed ($r_s=-0.27$, $p<0.001$), with an average decrease in the AL of 1.8 s for each winning confrontation (Fig. 1-A). No significant correlation between the number of exposures to aggressive confrontations and the AL of the non-aggressive rats was found during the screening session (Fig. 1-B). Moreover, the non-aggressive rats were not able to successfully defeat an intruder opponent, thus not meeting the criteria for aggressive behaviour.

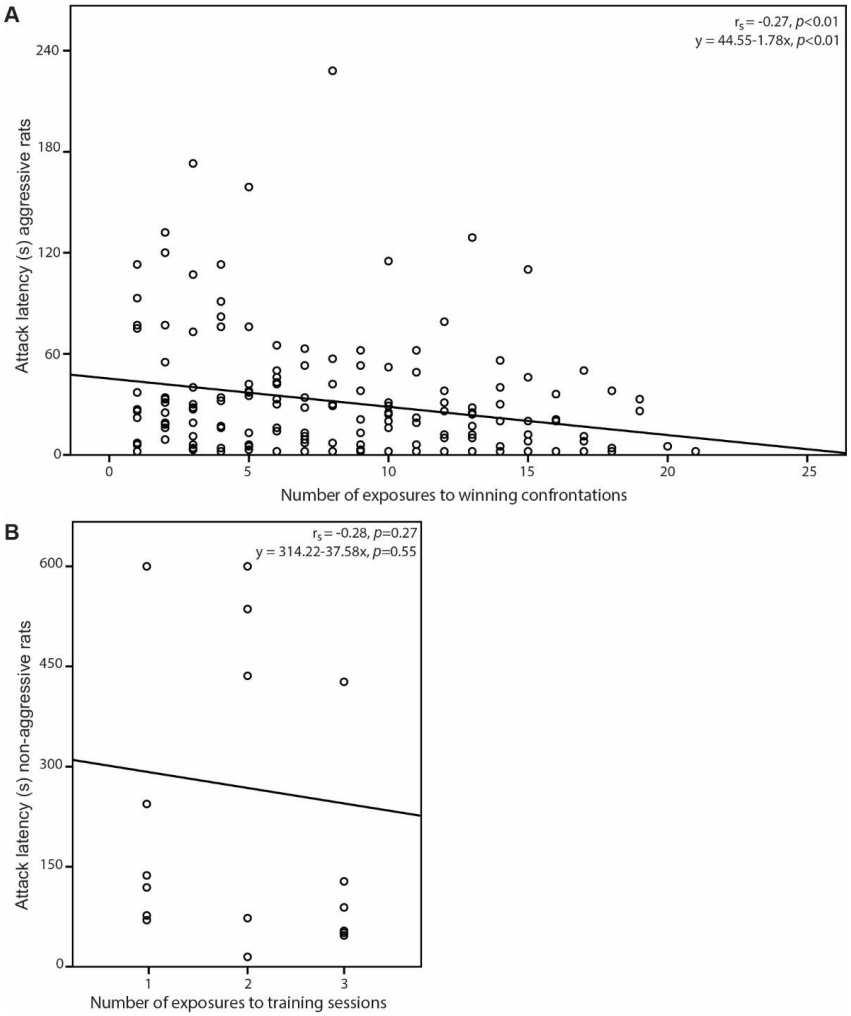

Figure 1 – A: Spearman correlation (r_s) between the number of the repetitive exposures to aggressive social conflicts and the attack latency (AL) in aggressive Long Evans (LE) rats (n=10). **B:** Spearman correlation (r_s) between the number of training sessions and the AL of the non-aggressive rats (n=6).

PET imaging of D2 receptor availability

A representative PET image of ^{11}C-raclopride PET in non-aggressive rats and aggressive rats is displayed in Fig. 2-A. The tracer binding in the investigated brain regions, calculated using the SRTM compartmental model, differed significantly between groups and brain regions (Fig. 2-B). Aggressive LE rats displayed a significantly higher BP_{ND} the NAc than non-aggressive rats (1.14, IQR 1.01 – 1.28 *vs.* 0.83, IQR 0.77 – 1.03, p=0.007). The same pattern was observed for the CPu, with an increased BP_{ND} in

aggressive as compared to non-aggressive rats (2.26, IQR 2.23 – 2.40 *vs.* 1.98, IQR 1.66 – 2.09, $p<0.001$). Time-activity curves of the NAc, CPu and cerebellum of non-aggressive and aggressive LE rats are presented in Fig. 2-C and D.

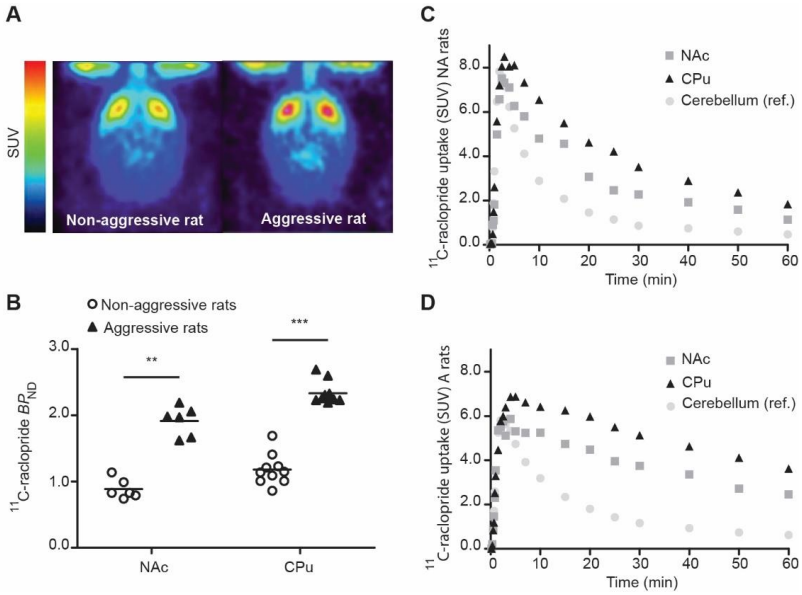

Figure 2 – A: ^{11}C-raclopride representative PET imaging of a non-aggressive rat (NA) and an aggressive rat (A). **B:** Graphical comparison of ^{11}C-raclopride binding potential (BP_{ND}) in the nucleus accumbens (NAc) and caudate and putamen (CPu) between non-aggressive and aggressive Long Evans rats. **C:** Representative ^{11}C-raclopride PET time-activity curves (TACs) of a non-aggressive Long Evans rat, and **D:** of an aggressive Long Evans rat.

Finally, we tested whether individual differences in aggressive temperament are related to D2 receptor availability. Therefore, the AL of the last aggressive exposure was correlated with the BP_{ND} of the NAc and CPu for non-aggressive and aggressive rats. In the NAc (Fig. 3-A), no significant correlation between the BP_{ND} of non-aggressive rats and the AL was found (r_s=0.43, p=0.40). However, a strong and significant negative correlation was observed for aggressive rats (r_s=-0.72, p=0.02). In the CPu (Fig. 3-B), no correlations were found at all for both non-aggressive and aggressive rats (r_s=0.26, p=0.62 and r_s=-0.21, p=0.56; respectively). Also, in order to evaluate if repetitive exposure to winning experiences might alter D2 receptor properties, the three first averaged AL measured during aggressiveness screening were correlated with the BP_{ND} of both NAc and CPu. No significant correlations were found in the NAc for the non-aggressive (r_s= 0.09, p=0.87) and aggressive rats (r_s=-0.24, p=0.51). Similarly, no significant correlations

were found in the CPu for the non-aggressive (r_s= 0.37, p=0.47) and aggressive rats (r_s= 0.15, p=0.68).

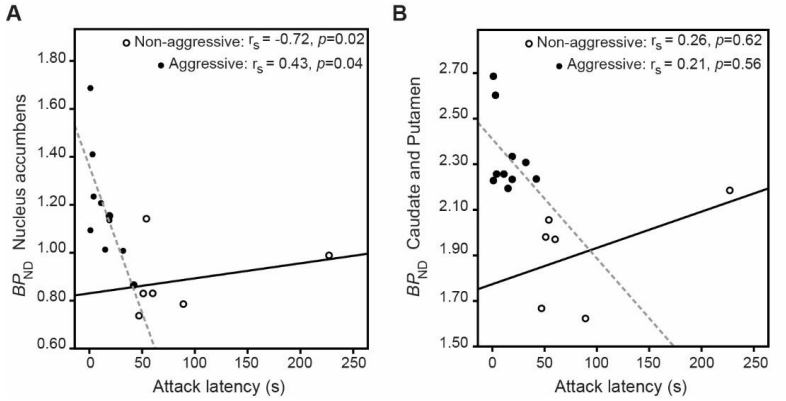

Figure 3: Spearman correlation (r_s) between the last attack latency (AL) and the binding potential (BP_{ND}) of ^{11}C-raclopride PET in the **(A)** nucleus accumbens (NAc) and **(B)** caudate and putamen (CPu) of non-aggressive and aggressive Long Evans (LE) rats.

Discussion

In the present study we have demonstrated through ^{11}C-raclopride PET that aggressive LE rats exposed to repeated winning confrontations display higher levels of D2 receptors in the striatal brain area, when compared to non-aggressive LE rats. This was followed by a decrease in the AL of aggressive LE rats relative to the number of exposures to successful aggressive confrontations.

Our results are in accordance with previously obtained data in rodents and non-human primates. In a study conducted by Jupp et al. (22), higher levels of D2/D3 receptors and dopamine transporter and decreased DA levels were found in the dorsal striatum and NAc in dominant rats than in subordinate rats. Also, in non-human primates, socially housed dominant monkeys that were engaged in aggressive behaviour had increased levels of D2 receptors in the basal ganglia when compared to subordinates, as was observed with ^{18}F-fluoroclebopride PET (23). Nader et al. (24) confirmed this finding in dominant female cynomolgus monkeys. Taking these data together, the higher levels of tracer binding in the aggressive dominant rats in our study indicates increased levels of D2 receptors and/or decreased levels of DA. These results suggest that social dominance status and/or level of aggressiveness should be considered as an important variable underlying individual variation in striatal D2 receptors.

Furthermore, a significant negative correlation between the number of aggressive confrontations and the AL was found. In this context, each exposure to winning confrontations might function as rewarding stimulus. The combination of the decreased AL and the increased tracer binding in the NAc (a brain area extensively associated with addiction (25)) after repetitive victorious aggressive confrontations seems to suggest that repetitive exposure to the rewarding effect of winning a social conflict could develop an "addictive-like" behaviour in conjunction with escalation of aggression. Additionally, an interesting finding arose from the correlation between the three first averaged AL and the BP_{ND} of the investigated brain regions, both in aggressive and non-aggressive rats. In contrast with our findings in the aggressive rats after repeated exposure to winning confrontations, we did not find any significant correlation between the three first averaged AL and brain regions in any group. This might suggest that exposure to repeated winning confrontations might alter the dopaminergic D2 receptor properties, resulting in higher BP_{ND} of ^{11}C-raclopride in the CPu and NAc of aggressive rats, but not in non-aggressive group.

In humans, impulsive violence is the most frequent form of violence with the greatest need for effective and evidence-based treatment (26). In vulnerable individuals, exposure to emotional provocative situations (e.g. drugs of addiction) leads to a weakening of control due to conditioned learning, resulting in impulsivity and compulsivity. Over time, individuals become conditioned to having violent reactions to provocative stimuli so that eventually such behaviour becomes automatic and a compulsive habit (Pavlovian conditioning) (25). This hypothesis of the evolution of violence into a habit or "addiction" might be comparable to how drugs of abuse lead from a single rewarding experience to a compulsive drug-seeking behaviour (26).

The present study has some limitations. Unfortunately, we were not able to conduct immunohistochemical analysis in brain samples in order to discriminate whether the increase in BP_{ND} of ^{11}C-raclopride in the NAc of aggressive rats was due to an increase in the D2 receptor levels or decrease in DA release. Moreover, no challenge with a dopaminergic psychostimulant (e.g. cocaine, amphetamines) was performed with the objective to evaluate vulnerability to DA reinforcers.

In conclusion, we were able to demonstrate increased D2 receptor levels in the NAc of aggressive dominant rats exposed to repetitive winning confrontations. The repetitive and habit-forming nature of aggressive winning of social conflicts might lead to escalated forms of aggression. Further studies are needed to corroborate our findings.

However, novel treatment strategies which targets the dopaminergic system and the restoration of the inhibitory controls might be of interest to decrease violence in society.

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Disclosure / Conflict of interest

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